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Apparent Dependence of Transformation on the Stage of Deoxyribonucleic Acid Replication of Recipient Cells

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We would like to open this symposium dealing with the entrance of foreign nucleic acids into bacterial cells in an unorthodox way, namely, by presenting data that suggest that sometimes, perhaps quite frequently, deoxyribonucleic acid (DNA) may not really enter in a free form into cells into which it transfers information, at least not in the sense of an actual penetration of free DNA into the cytoplasm. Our results suggest that, as already suspected by some other investigators (4, 5, 16, 18), the critical event, namely, the interaction between donor DNA and the recipient's DNA, may occur in the bacterial membrane rather than in the cytoplasm. The pertinent data were collected in studies on transformation of *Bacillus subtilis* in which recipient cell populations with synchronized DNA replication were employed.

This synchronization of the replication of DNA in the recipient cells was an accidental but, as it turned out, important by-product of the use of spores for initiating cultures that led to populations containing competent recipients for transformation. A procedure based in part on the method of Ephrati-Elizur (8) was employed. About 10^5 spores derived from polyauxotrophs of strain 168 were suspended in minimal medium supplemented with 0.1% yeast extract and incubated at 37 C. After incubation for 18 hr, the cells were diluted 1:10 into fresh medium of identical composition. Competence for transformation appeared in this medium about 60 min later and lasted for about 3 hr. A large proportion of bacteria were in a synchronized state of DNA replication during this period. This synchronization was confirmed by a test based on Sueoka and Yoshikawa's (14) results which employed transforming DNA preparations extracted from the cell population at various times after the initial culture had been transferred. In such tests, a sequential enrichment (up to 40%) of replicated markers, according to their location on the *B. subtilis* chromosome, was observed.

Such spore-derived, partially synchronized, and competent cell populations were used as recipients in studies in which transforming DNA, extracted from *B. subtilis* 168^+ by the method of Anagnostopoulos and Spizizen (2), was added in a concentration of 5 μg/ml at different times after the beginning of the period of competence.

As shown in Fig. 1, a striking and differential cyclic shift in the relative frequency of transformation was obtained when transformation events involving three nonlinked markers, threonine, methionine, and leucine, were scored. Since the general level of competence of the recipient cells changed during the 120 min of observations shown in Fig. 1, all results were calculated in terms of the percentage of a given transformed class among all transformants. Thus, the data shown are independent of the actual degree of competence of the recipient population at time of DNA addition. It can be noted that the sequence of the peaks for transformation frequencies of the three markers corresponds to their known relative locations on the *B. subtilis* linkage map.

When these results are replotted as rate of increase of transformants versus Δt (change in time) and normalized with respect to the total number of transformants (Fig. 2), the data become even more meaningful. It can be seen that the rates of increase for the three nonlinked markers differ strikingly. Initially, the rate is highest for threonine and leucine, and is very low for methionine; at a later time interval, this relationship is reversed. Figure 3 shows results of a test in which arginine, leucine, and methionine served as markers, and Fig. 4 shows results from an experiment in which transformation events involving the adenine, leucine, and methionine loci were scored.

In all of these tests, the temporal sequence in attainment of the peak of transformation frequency for a given marker corresponded to its known relative location on the linkage map; in other words, the efficiency with which a DNA fragment was integrated into the recipient genome was found to be related in some way with the chromosomal replication map of *B. subtilis*. In fact, it became possible to construct a linkage map

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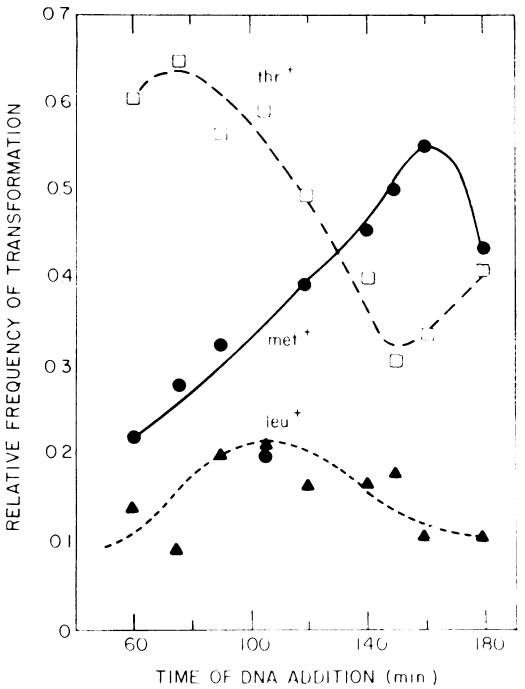

FIG. 1. *Fluctuation of the relative frequency of transformation of three nonlinked markers (thr, met, leu) as a function of time of addition of DNA to recipient cells. Recipient culture was initiated with spores, and time of DNA addition represents minutes after transfer of an 18-hr culture to fresh culture medium.*

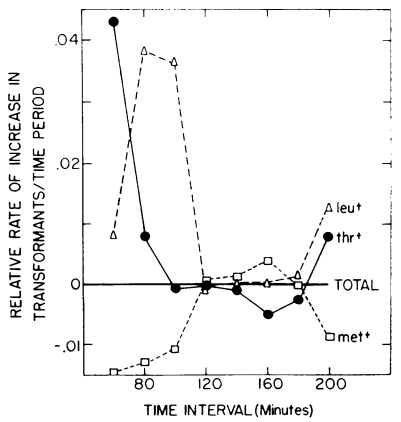

FIG. 2. *Changes in the relative rate of increase in transformants, involving the three nonlinked markers threonine, methionine, and leucine, as a function of DNA addition at one of several successive 20-min periods starting 60 min after transfer of a spore-derived, 18-hr culture to fresh medium. All points were calculated in terms of a normalized value for the total number of transformants.*

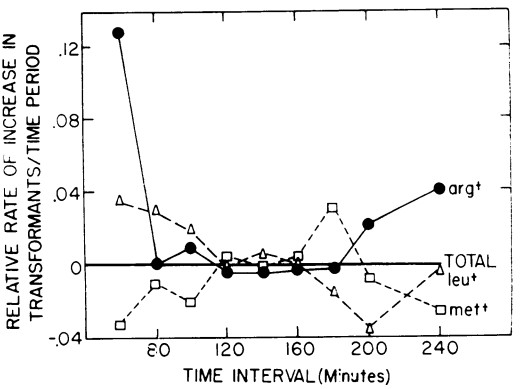

FIG. 3. *Changes in the relative rate of increase in transformants, involving the three nonlinked markers arginine, leucine, and methionine, as a function of DNA addition at one of several successive 20-min periods starting 60 min after transfer of a spore-derived, 18-hr culture to fresh medium. All points were calculated in terms of a normalized value for the total number of transformants.*

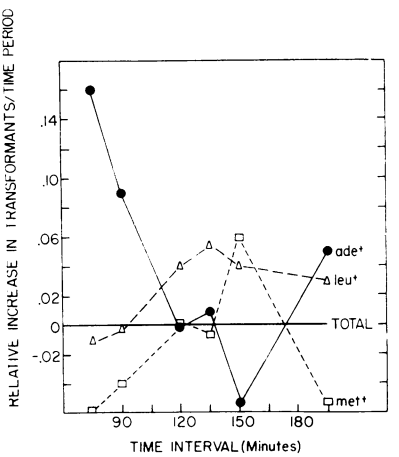

FIG. 4. *Changes in the relative rate of increase in transformants, involving the three nonlinked markers adenine, leucine, and methionine, as a function of DNA addition at one of several successive time periods starting 75 min after transfer of a spore-derived, 18-hr culture to fresh medium. All points were calculated in terms of a normalized value for the total number of transformants.*

merely by scoring relative transformation frequencies after different times of addition of transforming DNA to a competent culture. The resulting map agreed with previously published data of others who used, of course, entirely different procedures [e.g., Sueoka and Yoshikawa (14)].

It was also shown that linked markers (i.e.,

cistrons that are so close together that they are likely to end up on the same piece of DNA in the transforming DNA preparation) showed idential fluctuations when DNA was added at different times to the synchronized recipient culture (Fig. 5). The markers *tyr* and *aro* are known to be linked and can be seen to behave alike, in contrast to the nonlinked leucine marker.

How can these data best be interpreted? We have previously suggested (9; R. J. Erickson, W. Braun, and O. J. Plescia, Bacteriol. Proc., p. 53, 1967) that these results can be explained on the basis of Bodmer's (4) original suggestion that integration of DNA will occur at the point of DNA replication. We already know that transforming DNA associates itself with the membrane and therefore probably also with a point at which, according to data of others (*see* 10), DNA replication occurs. At this replication point, transforming DNA may be integrated into (or better, exchanged for) a homologous region once this region arrives at the replication point, or, alternatively, a homologous region, when it arrives at the replication point, may drag the transforming DNA into the cell and exchange may occur subsequently. In any event, interactions prerequisite to exchange would occur in the membrane at the point of DNA replication, and it is, of course, tempting to suggest that the availability of single-stranded regions in the DNA of

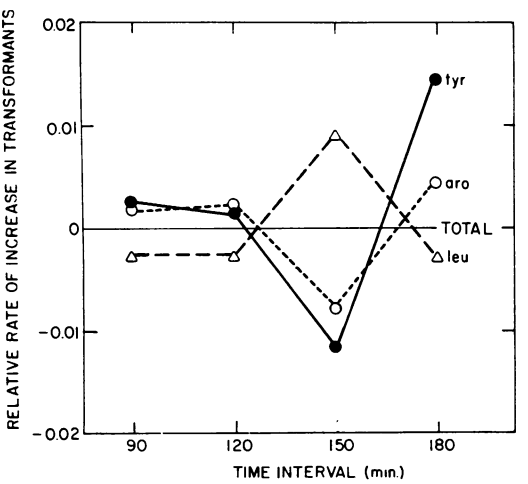

FIG. 5. *Changes in the relative rate of increase in transformants, involving two linked (tyrosine, aromatic) and one nonlinked (leucine) markers, as a function of DNA addition at one of several successive 30-min periods starting 90 min after transfer of a spore-derived 18-hr culture to fresh medium. All points were calculated in terms of a normalized value for the total number of transformants.*

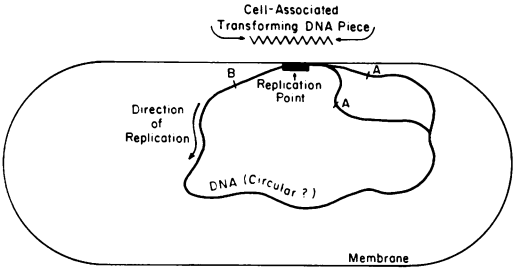

FIG. 6. *Model of some suggested stages in transformation. For details, see text.*

the recipient cell at this point can lead to interactions involving hydrogen bonding with the single-stranded donor DNA. If one now assumes a gradual decay of the membrane-associated DNA with time, then one can explain the results shown in Fig. 1–5 by the scheme illustrated in Fig. 6. As already stated, the competent recipient cells in our tests appear to be fairly well synchronized in respect to DNA replication and possibly, but not necessarily, may be at an identical stage of temporarily suspended chromosomal replication (4). Thus, taking a recipient population at a time when the DNA region controlling marker B happens to be near the replication point will assure a high relative frequency of transformation for this marker, in contrast to a distant marker A, which will not reach the replication point until some time later. If the recipient population is exposed to DNA at a later time when region A has become proximal to the replication point and B has become distal, the conditions would be reversed and the relative frequency of transformation for A would be higher than that for B. This is exactly what the experimental data showed, and, as already mentioned, by comparing relative frequencies of transformation for nonlinked markers when DNA was added at different times to the recipient culture, it was in fact possible to estimate the relative positioning of these markers on a circular DNA replicon.

One other series of tests has supported the scheme shown here. One would anticipate that a co-transfer of two nonlinked markers would be less likely to occur when one of the marker regions has passed the replication point. This was found to be the case. Table 1 shows the number of double transformants observed when wild-type DNA was added to *thr⁻ met⁻ leu⁻* recipients at either one of two different times after transfer of the spore-derived recipient culture to fresh medium. Clearly, the frequency of double transformation ["congression" (13)] of any two of the three nonlinked markers shifts strikingly as a

TABLE 1. *Influence of time of DNA addition on the frequency of double transformations involving two nonlinked markers*

Time of DNA addition	No. of double transformants per ml		
	thr$^+$ *met*$^+$	*thr*$^+$ *leu*$^+$	*met*$^+$ *leu*$^+$
a (120 min)	480	260	150
b (170 min)	90	230	170

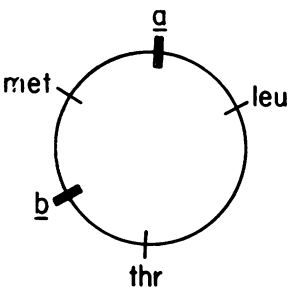

FIG. 7. *Location of markers on the B. subtilis chromosome. The letters a and b refer to times of DNA addition given in Table 1.*

function of the time of addition of the transforming DNA to the competent, synchronized recipient culture. The results can be explained easily by reference to the known location of the markers on the *B. subtilis* chromosome (*see* Fig. 7) and the anticipated progression in replication of chromosomal DNA from time *a* to time *b*. At time *a*, the leucine cistron presumably has passed the replication point; thus, the relative frequency of congression decreased in the order TM > TL > ML. At time *b*, the methionine cistron has passed the replication point and the decreasing frequency of congression has become TL > ML > TM. Thus, in accordance with our model, two markers approaching the replication point will be integrated into the same recipient cell with a high frequency, whereas the frequency becomes low once one of the two markers has passed the replication point. Incidentally, such data indicate that the co-transfer index for nonlinked markers is not constant in *B. subtilis*, being dependent on the stage of DNA synthesis in the recipient cell, and care must be taken in the use of such values for the estimation of cellular events.

Our conclusion that DNA uptake and integration are dependent on DNA replication of the recipient cell (Erickson et al., Bacteriol. Proc., p. 53, 1967) is supported by recent data of W. G. Cooper and J. E. Evans (Biophys. Soc. Abstr., p. A-79, 1968). However, it is in conflict with some recent interpretations by L. J. Archer and O. E. Landman (*submitted for publication*), who based their conclusions on some very ingenious experiments in which chromosome replication of an auxotroph of *B. subtilis* was arrested at the terminus by exposure of the cells to an environment devoid of tryptophan and thymine. When such arrested cells were then exposed to transforming DNA in the presence of tryptophan, a condition that does not reinitiate chromosome replication, competence developed, as revealed by transforming events assayed during subsequent incubation in complete medium. Furthermore, protoplasting of the arrested cells 25 min after DNA addition and exposure of the protoplasts to deoxyribonuclease did not produce a significant shift in the relative frequency of entry of different nonlinked markers between synchronously arrested cells and nonsynchronized control cultures. On the basis of such data, Archer and Landman concluded that "a mechanism of donor DNA entry involving homology at the replication fork is excluded."

On the basis of some very recent results obtained by R. J. Erickson and F. E. Young (*in preparation*), it is possible to suggest that the data of Archer and Landman are not necessarily in conflict with our conclusions. We have previously found (6, 9; Erickson et al., Bacteriol. Proc., p. 53, 1967) that transformation can still be inhibited by antibodies to DNA or to defined polynucleotides at a time when deoxyribonuclease no longer affects transforming DNA. Erickson and Young, working with ^{131}I-labeled antibodies and deoxyribonuclease, have now demonstrated that both antibody and deoxyribonuclease are bound maximally to *B. subtilis* cells just prior to the peak of competence. The binding appears to be electrostatic in nature and involves the cell membrane. Despite the identity of kinetics of uptake of the antibody and the nuclease, the antibody retains the ability to interact with transforming DNA at membrane sites, whereas the enzyme, though apparently active when membrane-bound (3, 11), no longer affects transforming DNA. These findings suggest that transforming DNA, which presumably has become single-stranded after traversing the cell wall barrier (15), may associate itself with the membrane in such a fashion that the phosphodiester bonds are protected, whereas the hydrophobic bases are exposed. One can, therefore, suggest that in the Archer and Landman studies membrane-bound, deoxyribonuclease-insusceptible DNA may have become integrated into the recipient's DNA when DNA replication was resumed at a later time, i.e., during subsequent incubation in complete medium. The only additional assumption that must be made is that, under the conditions employed by Archer and Landman (in contrast to those employed by Cooper and Evans and by us),

membrane-bound DNA persisted for a prolonged period of time. Another possibility is that resumption of DNA replication after chromosome arrest may lead to multiple membrane-associated replication sites, a possibility that has already been considered by Archer and Landman (*submitted for publication*).

Obviously, the term "competence" has been employed to describe the functioning of a multitude of sequential steps that are involved in events that transpire during the period between exposure of a bacterium to transforming DNA and the appearance of some of this DNA in the genome of the recipient cell. Specific receptor molecules on cell envelopes appear to play a part (1, 7), and autolytic enzymes have been implicated in the formation of holes in the cell wall (17). Once the DNA has penetrated into the space between wall and membrane, it may be denatured prior to the attachment to the membrane. After attachment [which, as indicated by the data of Kammen et al. (11), is probably a reversible process and may subject detached DNA to degrading enzymes], polynucleotide regions with fair homology to DNA regions of the recipient may be hydrogen-bonded with single-stranded regions of replicating DNA, permitting cross-membrane transport and either immediate or subsequent integration. The necessary homology would be lacking in the case of truly foreign DNA, thus preventing this entrance mechanism. However, foreign DNA would be able to occupy critical membrane sites, an event that can be held responsible for the well-known inhibitory effects of foreign DNA on transformation. Pairing of donor and recipient DNA has previously been suggested by Pittard and Walker (12). They noted that the presumably obligatory reaction between the leading edge of the donor DNA molecule and the recipient DNA leads to an increased frequency of recombination in this region of the recipient's DNA, and, consequently, the relative frequency of recombination is not constant over the entire length of the DNA molecule. It may well be that all recombination events in bacteria, regardless of the specific mechanism of transfer involved, involve entrance of donor DNA through replication sites in the membrane. How many such sites there may be per cell remains an open question; available evidence and published speculations (4, 5, 16; Archer and Landman, *submitted for publication*) suggest that there may be, at least under certain conditions, quite a number of such sites.

SUMMARY

Addition of transforming DNA to *B. subtilis* cells in which DNA replication has been synchronized results in differential cyclic shifts of frequency of transformation for different nonlinked markers. These results have been interpreted as reflecting a dependence of DNA uptake and integration on events occurring at the membrane site of replication of the recipient cell's DNA. Studies on shifting frequencies of double transformations ("congression") in synchronized recipients, and preliminary observations with radioactively labeled deoxyribonuclease and antibodies to DNA, have supported the conclusion that transforming DNA may enter into the cell's interior only via a direct association (hydrogen-bonding?) with partially homologous regions of replicating DNA of the recipient.

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